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Role of the $\alpha_v\beta_3$ integrin in human melanoma cell invasion

(vitronectin receptor/vitronectin/signal transduction/collagenase IV)

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ABSTRACT **The human melanoma cell line A375M expresses the vitronectin receptor ($\alpha_v\beta_3$ integrin) on its cell surface. Treatment of A375M cells with either polyclonal or monoclonal anti-$\alpha_v\beta_3$ antibodies resulted in stimulation of invasion through basement membrane matrices *in vitro*. Similar treatment of these cells with a monoclonal anti-α_v antibody, which does not inhibit the adhesive function of the $\alpha_v\beta_3$ antigen, also stimulated invasion; however, anti-β_3 antibody treatment had no effect. Furthermore, pretreatment of the cells with vitronectin or addition of vitronectin to the basement membrane matrix also resulted in stimulation of invasion. Similar treatments with fibronectin receptor antibody or fibronectin had no effect on invasion. Analysis of type IV collagenase expression in cells treated with anti-$\alpha_v\beta_3$ antibody showed higher levels of both the secreted 72-kDa enzyme and its mRNA. Signal transduction through the $\alpha_v\beta_3$ integrin could underlie the elevated expression of metalloproteinase and the enhanced invasion of A375M cells through basement membrane matrices.**

The three-step model for tumor cell invasion describes the initial process of the complex metastatic cascade in relatively simple terms: cell attachment to the extracellular matrix, proteolytic dissolution of the matrix, and movement of cells through the digested barrier (1). This process can occur repeatedly during the course of intra- and extravasation and can result in metastases at sites distant from the original tumor. The integrins, which are a family of cell-surface proteins that mediate cell–substratum and cell–cell adhesion, are important mediators of some of the interactions that constitute the metastatic process. The integrins are heterodimers of noncovalently linked α and β subunits, each of which is a transmembrane protein (2–4). Eleven α and six β subunits have been identified; in various combinations they can produce at least 16 distinct integrins (5–7). Furthermore, a β subunit can associate with multiple α subunits, and a single α subunit can become paired with more than one β subunit. In addition, more than one ligand can be bound by most integrins (5–8).

While *in vitro* and *in vivo* comparisons between normal and malignant cells suggest that changes in integrin expression accompany malignant transformation (9), little correlation exists between the altered pattern of integrin expression and tumorigenesis (7). However, elevated expression of the $\alpha_5\beta_1$ fibronectin receptor integrin is associated with lowered tumorigenicity (9, 10–12). Moreover, inhibition of integrin functions has suggested that integrins are important components of the metastatic process (7, 13–18). Earlier, correlative observations suggest that the $\alpha_v\beta_3$ integrin (also known as the vitronectin receptor) is one of the integrins that plays a role in tumorigenicity and metastasis. Thus, elevated expression of this integrin is associated with invasive melanoma *in vitro* (7). We have explored the relationship between the function and expression of the $\alpha_v\beta_3$ integrin in A375M human melanoma cells and the ability of these cells to invade *in vitro* by modulating this integrin with either antibodies or its ligand vitronectin. We find that such treatments can enhance invasiveness of these cells and propose that a probable mechanism contributing to this enhancement is the induction of the matrix-degrading protease, type IV collagenase.

MATERIALS AND METHODS

Cell Culture. The A375M human melanoma cell line was a kind gift from I. J. Fidler (The University of Texas, M.D. Anderson Hospital and Tumor Institute, Houston, TX) and was maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum (Gemini BioProducts, Calabasas, CA) as described (19). The cell cultures were determined by the Gen-Probe rapid detection system (Fisher) to be free of mycoplasma contamination.

Antibodies and Proteins. Rabbit antisera prepared against the $\alpha_v\beta_3$ (antiserum 237; refs. 20 and 21), $\alpha_5\beta_1$ (antiserum 255; ref. 10), and $\alpha_3\beta_1$ (22, 23) integrins, and against the cytoplasmic peptides of the β_3, α_v, and α_5 integrin subunits (10, 21) have been described as have monoclonal antibodies (mAbs) recognizing the $\alpha_v\beta_3$ (LM609; refs. 24 and 25) and the α_v (21), β_1 (26), and β_3 integrin subunits. Antibodies were affinity purified from the anti-$\alpha_v\beta_3$ and anti-$\alpha_5\beta_1$ antisera by absorption onto and elution from the appropriate purified integrin (20). Vitronectin and fibronectin were isolated from human plasma as described (27, 28).

Indirect Immunofluorescence. A375 cells were grown on coverslips, fixed with 3.7% formaldehyde, washed with 0.1% Triton X-100 in phosphate-buffered saline (without Ca^{2+} and Mg^{2+}) (PBS) and then treated with 1 μg of affinity-purified rabbit antibody per ml in PBS for 1 hr. After washing, the cells were treated with the secondary rhodamine-conjugated goat anti-rabbit IgG antibody for 1 hr and washed; the coverslips were mounted on microscope slides with Gelvatol (Monsanto). Controls were incubated with the goat anti-rabbit antibody only. The slides were viewed under a Zeiss microscope equipped for epifluorescence using a Zeiss automatic rhodamine filter set and a 50-W DC illumination system (Opti-Quip).

Immunoprecipitation. Cells were harvested with 2 mM EDTA, washed in PBS, and surface iodinated as described (10, 23). Labeled cells were extracted in Tris-buffered saline (pH 7.5) containing 50 mM octyl β-D-glucopyranoside, 1 mM

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Abbreviations: MICS, membrane invasion culture system; mAb, monoclonal antibody.

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phenylmethylsulfonyl fluoride, and 1 mM $MnCl_2$. Equivalent amounts of radioactivity were added to Eppendorf tubes containing anti-rabbit IgG-agarose (Sigma) and rabbit anti-integrin subunit antibodies or anti-mouse IgG-agarose and monoclonal anti-integrin antibodies. Immunoprecipitates were washed, solubilized, and electrophoresed in a SDS/7.5% polyacrylamide gel. After drying, the gel was autoradiographed against Kodak X-Omat film overnight at −70°C.

***In Vitro* Invasion and Adhesion Assays.** The *in vitro* invasion was performed in the membrane invasion culture system (MICS) as described (19, 29). Briefly, after filling the lower wells of the MICS with DMEM plus 10% NuSerum (Collaborative Research), the MICS was assembled by using a polycarbonate filter containing 10-μm pores coated with Matrigel (Collaborative Research) as the intervening invasive barrier. Cells (10^5) were seeded into the upper wells (measuring 1.3 cm in diameter) in the same medium and in a randomized manner across the chamber. The chamber was then placed in a 37°C incubator with 5% CO_2/95% air for 72 hr. After 72 hr, the cells recovered from beneath the membrane were counted and percentage invasion was determined compared to the total number of cells originally seeded after correcting for cell proliferation. The invasive potential of the untreated control cells was normalized to 100% invasion and the invasive potential of the treated cells was determined as a percentage of the control.

The adhesion of human melanoma cells to vitronectin, fibronectin, and laminin in the presence of antibodies to various anti-integrin subunits was done as described (13) with experiments performed in triplicate and duplicated.

Detection of Vitronectin in Matrigel. Matrigel was added to sample buffer (1:5) and heated at 100°C for 5 min. The sample was then electrophoresed in a SDS/10% polyacrylamide gel and electrophoretically transferred to a sheet of Immobilon transblot membrane (Millipore). Subsequently, the blot was probed with anti-mouse vitronectin antiserum followed by a horseradish peroxidase-conjugated secondary antibody to detect for the presence of vitronectin on the blot. A second sample was titrated on a SDS/10% polyacrylamide gel against known concentrations of vitronectin to help establish the concentration of the protein identified as vitronectin on the immunoblot.

Analysis of Type IV Collagenase. Northern blot analysis of collagenase IV mRNA expression was performed on untreated control A375M cells or on A375M cells treated overnight with various antibodies at 1 μg/ml. Poly(A)-selected RNA (1 μg per lane) was fractionated on a 2.2 M formaldehyde gel, capillary blotted to GeneScreen*Plus* nylon membrane (DuPont/NEN), and probed with ^{32}P-labeled cDNA for the 72-kDa human collagenase IV gene (pH3A cDNA). The blots were exposed to Kodak XAR-5 film for 72 hr and the relative amounts of collagenase IV mRNA were determined by densitometric scanning with a model 620 video densitometer (Bio-Rad). The blot was reprobed with ^{32}P-labeled β-actin cDNA to determine whether a difference existed in mRNA loading.

Zymographic analysis of secreted levels of collagenase IV was performed as described (30, 31). The assays were initiated by treating cells in serum-containing medium for 30 min with various antibodies at 1 μg/ml before plating into tissue culture flasks. After 2 hr, the cells were washed and serum-free medium was added back to the cells along with fresh antibody. After 18 hr, the medium was removed from the cells and frozen at −80°C until it was concentrated 10-fold for zymography with a YM-30 membrane (Centricon, Amicon). The gels were photographed at 1:1 and the negatives were densitometrically scanned (as described above) to determine the relative densities of the zones representing the 72-kDa collagenase activity.

RESULTS

Integrins in A375M Cells. Indirect immunofluorescence microscopy of A375M cells treated with an anti-$\alpha_v\beta_3$ antibody followed by a secondary fluorescent antibody shows a punctate staining pattern typical for focal adhesion sites (Fig. 1). The presence of this and other integrins expressed on A375M cells was further demonstrated by immunoprecipitations performed on surface-iodinated, lysed cells. These data demonstrate that, in addition to $\alpha_v\beta_3$, A375M cells express at least the $\alpha_5\beta_1$ integrin, which serves as a receptor for fibronectin as well as, possibly, other β_1 integrins, which serve as receptors for interstitial collagens, type IV collagen, and laminin. Antibodies against the α_v subunit coprecipitated the β_3 subunit, indicating that the α_v subunit is associated with the β_3 subunit in these cells (Fig. 2). Furthermore, cell attachment assays established the ability of the A375M cells to attach to surfaces coated with each of these proteins (results not shown).

Effect of Anti-Integrin Antibodies on A375M Cell Invasiveness and Adhesion. When A375M cells were treated with either affinity-purified polyclonal anti-$\alpha_v\beta_3$ antibody or with a mAb that recognizes the intact $\alpha_v\beta_3$ integrin (mAb LM609), the ability of the cells to invade basement membrane matrices increased substantially (Fig. 3). An anti-α_v mAb (mAb 147), which does not interfere with the adhesive function of the $\alpha_v\beta_3$ integrin (see below), similarly enhanced the invasive ability of the cells. However, this effect was not seen when the cells were treated with a mAb to the β_3 subunit or with a polyclonal affinity-purified antibody to the fibronectin receptor (the $\alpha_5\beta_1$ integrin). These antibodies were tested at a concentration of 1.0 μg/ml. When additional concentrations of mAbs to the $\alpha_v\beta_3$ integrin and α_v subunit were tested (0.1 and 8.0 μg/ml), the smallest active dose was 0.1 μg/ml, while greater stimulation was seen with consecutively higher concentrations. None of the antibodies affected the attachment of the A375M cells to Matrigel (data not shown), although the polyclonal and monoclonal anti-$\alpha_v\beta_3$ antibodies inhibit the adhesive function of the integrins they recognize. In agreement with earlier results (24, 25), the mAb LM609 inhibited cell adhesion to vitronectin when used at 5 μg/ml. The anti-$\alpha_v\beta_3$ mAb (dilution 1:100) was also inhibitory, but the anti-α_v mAb (ref. 21; mAb 147; 5 μg/ml) was not. None of the antibodies inhibited attachment to laminin or fibronectin. The anti-β_1 mAb (ref. 26; P4C10) did not inhibit cell attachment to vitronectin, but it did inhibit attachment to both fibronectin and laminin (results not shown).

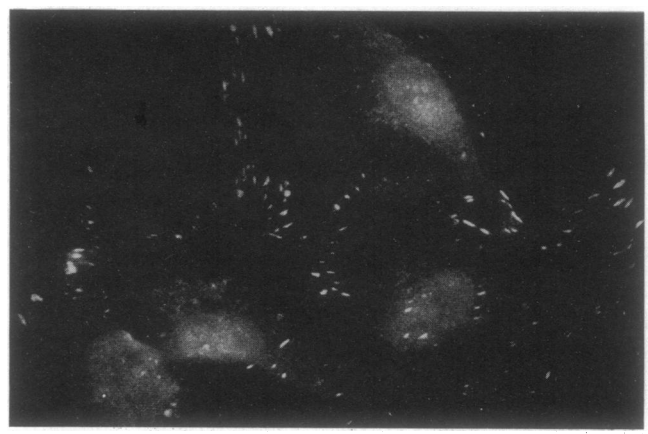

FIG. 1. Indirect immunofluorescence staining of A375M human melanoma cells for the $\alpha_v\beta_3$ integrin. The cells were incubated with affinity-purified polyclonal anti-$\alpha_v\beta_3$ antibody followed by a rhodamine-conjugated secondary antibody. Controls were incubated in goat anti-rabbit IgG antibody only and were negative for immunofluorescence staining (data not shown). (×460.)

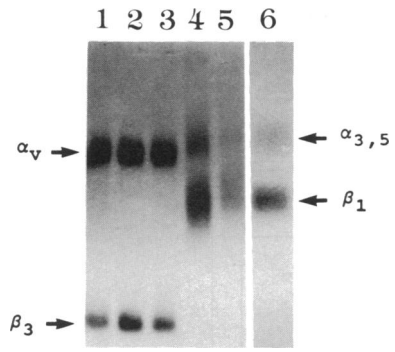

FIG. 2. Immunoprecipitation analysis of integrin subunits from A375M cells. Lanes: 1, affinity-purified rabbit anti-$\alpha_v\beta_3$ antibody; 2, anti-β_3 cytoplasmic peptide antiserum; 3, anti-α_v cytoplasmic peptide antiserum; 4, anti-β_1 monoclonal antibody (P4C10); 5, anti-$\alpha_3\beta_1$ polyclonal antiserum; 6, anti-α_5 cytoplasmic peptide antiserum.

Effect of Vitronectin on Invasion. The ability of the anti-α_v mAb 147 to stimulate invasion suggested that the $\alpha_v\beta_3$ integrin on A375M cells could affect the invasive process via mechanisms other than interactions involving cell matrix adhesion. We reasoned that if binding an inhibitory or noninhibitory antibody to the $\alpha_v\beta_3$ integrin could enhance invasion, the addition of the ligand for this receptor, vitronectin, might do the same. Because the invasion experiments were performed in the presence of 2% fetal calf serum, it was clear that some vitronectin would be contributed by the serum. At the concentration of calf serum used, this could be expected to be ≈5 μg/ml (32). We also examined the possibility that vitronectin could be a naturally occurring constituent of Matrigel. Immunoblotting with anti-mouse vitronectin antiserum showed that vitronectin was a component of the lot of Matrigel used in these experiments and that its concentration was ≈25 μg per ml of stock solution of Matrigel (13 mg of protein per ml).

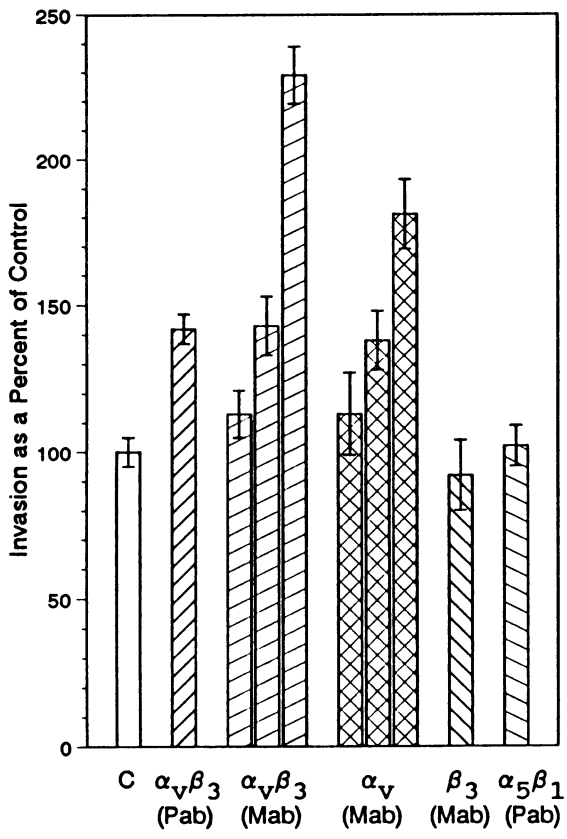

FIG. 3. Effect of anti-integrin antibodies on *in vitro* invasive potential of A375M human melanoma cells. A375M cells either were not treated with antibody (C, control cells) or were pretreated in suspension for 0.5 hr prior to the assay with 1 μg of the polyclonal anti-$\alpha_v\beta_3$ (Pab), polyclonal anti-$\alpha_5\beta_1$, or monoclonal anti-β_3 (Mab) antibodies per ml. Pretreatment of the cells with either the anti-$\alpha_v\beta_3$ or the anti-α_v mAbs was at 0.1, 1, or 8 μg/ml (left to right) prior to seeding into the MICS. Fresh antibody was added daily throughout the course of the 72-hr assay. The invasive potential of the untreated control cells was normalized to 100% invasion and the invasive potential of the treated cells was determined as a percentage of the control. The data shown are representative of three separate experiments for the anti-$\alpha_v\beta_3$ mAb and of two separate experiments for the affinity-purified rabbit anti-$\alpha_v\beta_3$ antibody. Error bars are based on n = 6 for each experiment and the SE was determined relative to control cells.

To increase the vitronectin concentration significantly in these experiments, cells were treated in suspension with 16 μg of soluble vitronectin per ml prior to the invasion assay or 32 μg of vitronectin per ml was added into the Matrigel used in the assay. In both cases, exposure to vitronectin resulted in an increase in cell invasion by up to 20–30% (Fig. 4).

Effect of Anti-Integrin Antibodies on Collagenase Production. It has been shown previously that rabbit synovial fibroblasts can be induced to express the metalloproteinases stromelysin and collagenase as well as accumulate the mRNAs for these enzymes by treating the cells with an antibody against a fibronectin receptor capable of blocking the initial adhesion of the cells to fibronectin (33). With this in mind, we sought to measure potential changes in type IV collagenase in response to interactions of the cells with anti-$\alpha_v\beta_3$ integrin antibodies. Type IV collagenase has been shown to be closely associated with the invasive and metastatic potential of a variety of tumor cells and to be critical for degradation of type IV collagen in basement membranes, including Matrigel (29, 34–38). Fig. 5*A* shows that treatment of A375M cells with the polyclonal anti-$\alpha_v\beta_3$ antibody resulted in an increase in the level of mRNA for the 72-kDa enzyme, while cells treated with the anti-$\alpha_5\beta_1$ antibody showed no change. The amount of mRNA is reported as arbitrary units relative to the level of the control lane and corrected for the amount of mRNA loaded per sample as determined by reprobing the blot for β-actin mRNA (Fig. 5*B*). In agreement with the mRNA data, zymography analysis showed elevated levels of the secreted 72-kDa enzyme in the cells treated with the anti-$\alpha_v\beta_3$ antibody (Fig. 5*C*). These results suggest that modulation of protease levels can result from perturbation of more than one integrin and that such a response can be mediated by different integrins in different cell lines.

DISCUSSION

We have shown that binding of anti-α_v antibodies to the $\alpha_v\beta_3$ integrin can trigger important biological responses in A375M human melanoma cells. Treatment of these cells with antibodies to $\alpha_v\beta_3$ caused an increase in their ability to invade basement membrane matrices concomitant with an increase in the expression and secretion of a matrix-degrading protease known as type IV collagenase.

A375M human melanoma cells express the $\alpha_v\beta_3$ integrin on their cell surface as determined by immunofluorescence microscopy and immunoprecipitation. The localization of the $\alpha_v\beta_3$ integrin in adhesion plaques and the ability of cells to attach to vitronectin indicate that the $\alpha_v\beta_3$ integrin is active in mediating the attachment of these cells to their substratum. Since the $\alpha_v\beta_3$ integrin binds to a number of Arg-Gly-Asp-containing proteins (6, 22, 24, 25, 39), and since Matrigel contains such proteins including vitronectin (in the present study), laminin and entactin/nidogen (40–43), it is possible that treatment with either anti-$\alpha_v\beta_3$ antibodies or vitronectin

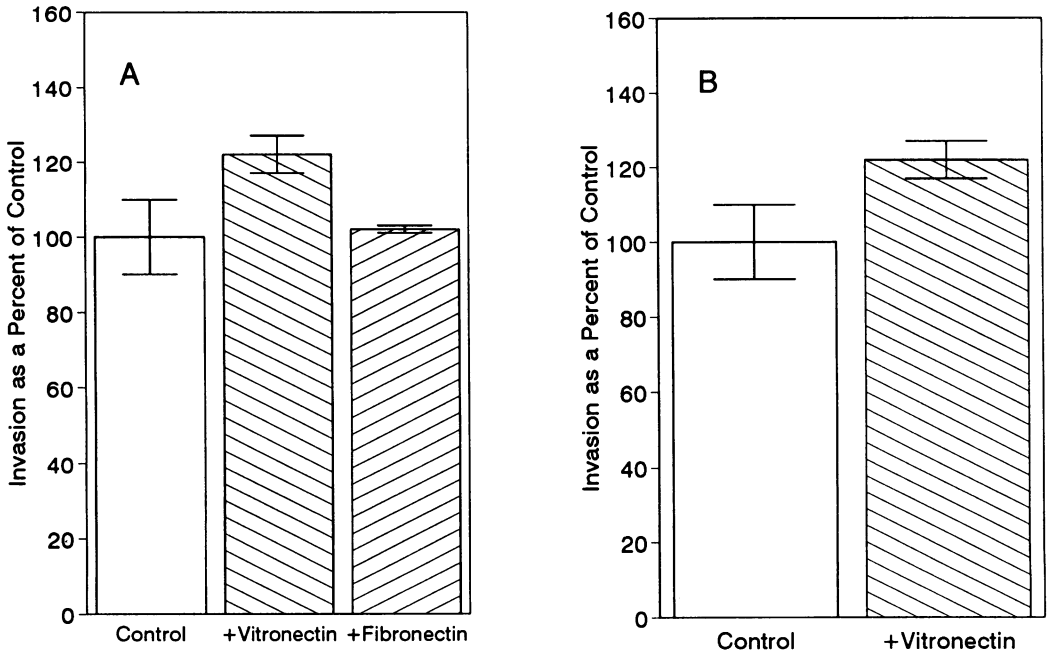

FIG. 4. (*A*) Invasion profile of A375M cells pretreated with either vitronectin or fibronectin. A375M cells were pretreated with 16 μg of either vitronectin or fibronectin per ml in suspension for 0.5 hr prior to seeding into the upper well of the MICS. Fresh vitronectin or fibronectin was added daily. The invasive potential of the control cells was normalized to 100% and the invasive potential of the treated cell is expressed as percentage invasion of control. Error bars represent SE and were determined relative to the control. (*B*) Invasion profile of A375M cells through a Matrigel-coated membrane containing an additional 32 μg of vitronectin per ml.

could have enhanced invasion by preventing binding of the integrin to its ligand(s) in the basement membrane matrix. Treatment of A375M cells with antibodies to the $\alpha_v\beta_3$, as well as with $\alpha_5\beta_1$ and $\alpha_3\beta_1$ integrins, resulted in a minor decrease in adhesion to Matrigel in a 1-hr assay (data not shown). However, this inhibition cannot explain the increased invasiveness observed over 72 hr in the MICS, because only the anti-$\alpha_v\beta_3$ treatment (but not treatment with anti-$\alpha_5\beta_1$ or anti-$\alpha_3\beta_1$) resulted in increased invasiveness and stimulated production of type IV collagenase by the A375M cells. Moreover, although both the polyclonal and monoclonal antibodies against the $\alpha_v\beta_3$ integrin do inhibit its adhesive function, we found that a noninhibitory anti-α_v antibody, mAb 147, was also capable of enhancing invasion. Finally, adding vitronectin to this system also increased the cells' invasiveness through Matrigel. The concentrations of vitronectin that promoted invasion were lower than those expected to inhibit cell adhesion (43). Thus, it appears that the invasiveness that results from ligation of the $\alpha_v\beta_3$ integrin can result from the binding of another macromolecule, either an antibody or soluble adhesion protein, to this integrin regardless of whether the ability of the integrin to mediate cell adhesion is affected. These results suggest that the increase in invasiveness is caused by a signal elicited upon ligation of the integrin.

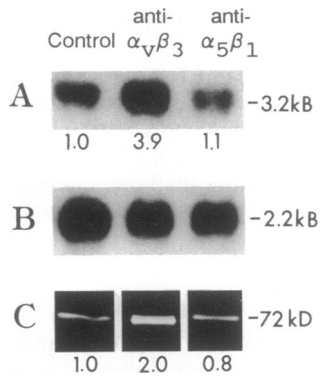

FIG. 5. (*A*) Analysis of collagenase IV mRNA expression by Northern blotting. Poly(A)-selected RNA from control A375M cells (lane 1) or A375M cells treated overnight with either a polyclonal anti-$\alpha_v\beta_3$ antibody (antibody 237; lane 2) or polyclonal anti-$\alpha_5\beta_1$ antibody (antibody 255; lane 3) (1 μg/ml). The blots were exposed to Kodak XAR-5 film for 72 hr and the relative amounts of collagenase IV mRNA were determined by densitometric scanning. The amount of mRNA is expressed as arbitrary units relative to the level in the untreated A375M cells and is corrected for the amount of mRNA loaded per sample as determined by reprobing the blot with ^{32}P-labeled β-actin cDNA and densitometrically scanning the resulting autoradiograph (*B*). (*C*) Zymographic analysis of collagenase expression. Cells were treated with antibodies and the culture medium was analyzed for type IV collagenase activity as described. Lanes: 1, medium from untreated cells; 2, culture medium from cells treated with the anti-$\alpha_v\beta_3$ antibody overnight; 3, culture medium from cells treated with the anti-$\alpha_5\beta_1$ antibody overnight. kB, kilobases.

The α_v subunit appears to be the subunit capable of mediating the putative invasion-enhancing signal, since an antibody against the β_3 subunit did not increase the invasiveness of the A375M cells. Furthermore, although the α_v subunit can combine with at least two other subunits, β_5 and β_1 (6), it is the LM609 mAb that is specific for only the $\alpha_v\beta_3$ integrin (24, 25), which elicits a signal that is responsible for an increase in melanoma tumor cell invasion.

Our results also provide some information on how the putative signal may enhance invasion by the A375M cells. We show that, in addition to increasing the invasive potential of the melanoma cells, treatment of these cells with antibodies to the $\alpha_v\beta_3$ integrin caused an increase in expression and secretion of type IV collagenase. The increase in collagenase production displayed the same specificity as the enhancement of invasion, since treatment of cells with antibodies to the $\alpha_5\beta_1$ fibronectin receptor, which is also expressed on the A375M cells, did not modulate the invasive activity of the cells or change levels of the secreted enzyme or mRNA levels of the collagenase. These data, therefore, strongly suggest a biological function specific for the $\alpha_v\beta_3$ integrin in melanoma cell invasion and implicate modulation of protease levels as

the effector mechanism in this function. The role of integrins in this process is supported by work demonstrating integrin-mediated stimulation of tyrosine phosphorylation in an intracellular 130-kDa protein (44). This integrin-mediated pathway may be critical for the transduction of information between the extracellular matrix and the cell interior, which further supports the importance of the tumor cell surface and its significance in the diagnosis (7, 45) and design of clinical therapies for metastatic disease (45, 46). The present biological findings contribute another perspective on the role of an adhesion receptor in a critical step in the metastatic cascade.

Note. After the acceptance of this manuscript, we immunoprecipitated the β_5 as well as the α_v integrin subunits from A375M cells by using a polyclonal anti-β_5 antibody. These data may offer additional insight into the involvement of integrins in tumor cell invasion.

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